

ORIGINAL ARTICLE

Performance of Alpha Fetoprotein in Combination with Alpha-1-acid Glycoprotein for Diagnosis of Hepatocellular Carcinoma Among Liver Cirrhosis Patients

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ABSTRAK

Tujuan: untuk menilai manfaat pemeriksaan alpha-1-acid glycoprotein (AAG) untuk mendiagnosis karsinoma hepatoselular (KHS), dan dalam kombinasi dengan alpha fetoprotein (AFP) sebagai bagian dari pemeriksaan rutin pada pasien sirosis hati. **Metode:** penelitian ini merupakan studi diagnostik dengan menggunakan desain potong lintang. Sebanyak 106 pasien diikutsertakan dalam penelitian ini. Data dasar seperti usia, jenis kelamin, kadar AFP, kadar AAG, pemeriksaan darah tepi, SGOT dan SGPT diambil secara kolektif dari pasien sirosis hati dengan atau tanpa KHS. Pemeriksaan kadar AAG serum dihitung dengan metode immunoturbidimetric assay dan pemeriksaan kadar AFP dengan enzyme immune assay. Analisis statistik dilakukan dengan menggunakan SPSS 13.0. Perbandingan data antar grup dilakukan dengan uji Mann-Whitney. Nilai diagnostik masing-masing penanda dibandingkan secara paralel pada kasus KHS. **Hasil:** analisis receiver operating characteristic (ROC) menunjukkan area di bawah kurva untuk kombinasi AFP dan AAG sebesar 88.1%, lebih tinggi dibandingkan AFP saja (86.2%) atau AAG saja (76.5%) dengan sensitivitas 83%, 73% dan 44% pada spesifisitas >80%. **Kesimpulan:** penelitian ini memperlihatkan perbandingan peran pemeriksaan AAG, AFP, dan kombinasi keduanya dalam mendiagnosis KHS pada pasien sirosis hati. Kombinasi AFP dan AAG dapat digunakan untuk deteksi dini dan penapisan KHS.

Kata kunci: alfa fetoprotein, alfa-1-acid glikoprotein, penanda biokimia, kanker hati.

ABSTRACT

Aim: to evaluate the use of alpha-1-acid glycoprotein (AAG) for diagnosing hepatocellular carcinoma (HCC), and to combine with alpha fetoprotein (AFP) as part of routine examination in liver cirrhosis patients. **Methods:** this is a diagnostic study using cross-sectional design. A hundred and six patients were included in this study. Baseline data such as age, gender, AFP, AAG, peripheral blood count, AST and ALT were consecutively collected from liver cirrhosis patients with or without HCC. Serum AAG were measured quantitatively using immunoturbidimetric assay and AFP with enzyme immune assay (EIA). Statistical analysis were done using SPSS 13.0. Data comparisons between group were done using Mann-Whitney test. Diagnostic performance for each marker alone was compared to the surrogate use of both markers (combined parallel approach) in HCC cases. **Results:** receiver operating characteristic (ROC) analysis showed that area under the curve for AFP-

AAG combination was 88.1% and higher than AFP only (86.2%) or AAG only (76.5%) with sensitivity of 83%, 73% and 44%, respectively, at specificity of >80%. **Conclusion:** our study showed that combination of AFP and AAG is superior than either marker alone in diagnosing HCC in liver cirrhosis patients. Combination of AFP and AAG may be used to prompt early diagnosis screening of HCC.

Key words: alpha fetoprotein, alpha-1-acid glycoprotein, biomarker, liver cancer.

INTRODUCTION

Hepatocellular Carcinoma (HCC) is the second most common cause of death from cancer worldwide, estimated to be responsible for nearly 746,000 deaths in 2012 (9.1% of the total) (globoscan, 2012).¹ Most of the HCC incidence occurred in developing countries, particularly in Asia and Africa which are susceptible to hepatitis B and hepatitis C. However, recent trends indicate that incidence of HCC in developed countries is increasing.² This could be explained by changes in the prevalence of hepatitis. Screening for HCC can lead to early diagnosis and treatment.

In most of the cases, HCC is diagnosed in its advanced stage that the treatment options are limited with lower survival rates. It means that diagnosing HCC in its early stage is of the utmost importance. Based on a systematic review, several serum biomarkers can be used to detect HCC.³ Alpha fetoprotein (AFP) is the most widely studied screening test for detecting HCC. The normal range for serum AFP levels is 10-20 ng/mL and a level > 400 ng/mL is usually regarded as diagnostic for HCC. Although Asia-Pacific Association for Study of the Liver (APASL) consensus used lower level of AFP (>200 ng/mL) for diagnosis of HCC, some reports have indicated that the high serum concentration of AFP correlates with the poor prognosis of HCC patients. However, two thirds of HCC patients with the nodule less than 4 cm have serum AFP levels less than 200 ng/mL and up to 20% HCC patients do not produce AFP.⁴

It has been recognized that AFP has a low sensitivity in detection of HCC. APASL guidelines 2010 do not recommend the use of AFP only for diagnosing HCC. Additional investigations are needed to establish the diagnosis.³ AAG is an acute phase protein with normal levels of 50-120 mg/dL. This serum

concentration increases in response to systemic tissue injury, inflammation or infection, and these changes in serum protein concentrations have been correlated with increases in hepatic synthesis.⁵ Raised levels of AAG were found in 80.6% of patients with hepatoma compared to 20% of patients with cirrhosis and only 5.7% of patients with hepatitis.⁶ Our group found that AAG was a potential biomarker in the diagnosis of HCC.^{7,8} In a study with 220 patients, of which 96 were control and 124 were HCC cases (61 were AFP-low and 63 were AFP-high), it was found that Alpha-1-acid glycoprotein (AAG) was more predictive of AFP-low HCC than of AFP-high HCC.⁷ The other study, combination between AFP and AAG could significantly improve the diagnostic accuracy. ROC analysis of AFP in combination with AAG yielded AUC (area under curve) value higher (0.943) than AFP alone (0.750) or AAG (0.907) alone.⁸

Our previous study showed AAG and AFP combination was a good biomarker for diagnosis of HCC. However, AAG determination in the previous study were measured with high performance liquid chromatography (HPLC) that can be done only in a research center and not routinely available in clinical laboratories. Furthermore, the subjects from previous studies did not specifically liver cirrhosis patients whom the risk of developing HCC are the greatest. In this study the quantitative measurement of serum AAG was done with nephelometric (immunoturbidimetric) assay in routine clinical laboratory. The subjects of this study were liver cirrhosis patients with HCC and compare it with liver cirrhosis patients without HCC. The objective of this study was to evaluate the use of AAG in routine laboratory as biomarker for screening of HCC among liver cirrhosis patients in combination with AFP.

METHODS

This is a cross-sectional study. The subjects of this study were liver cirrhotic patients, aged 18 years and older. Diagnosis of hepatocellular carcinoma in the subjects group were defined according to AASLD guidelines on hepatocellular carcinoma⁹ or by presence of liver nodule, AFP >200 ng/mL and supported with two imaging results with typical features of hepatocellular carcinoma. We included liver cirrhotic patients without liver nodule as control group in this study. A total of 106 patients who regularly followed up at Hepatobiliary Division, Department of Internal Medicine, Cipto Mangunkusumo Hospital, Indonesia between January to August 2013 who meet the criteria were consecutively enrolled and serum samples were collected. For each patient, clinical data, including age, sex, AFP value, AAG, hemoglobin, leukocyte, thrombocyte, AST, and ALT were obtained. Diagnosis of HCC relied on the presence of malignant liver nodule, as established on imaging techniques according to APASL criteria or by pathological analysis of liver biopsy, if necessary. Patients with other type of malignancies, without evident of liver cirrhosis, diagnosed with ongoing acute infections (pneumonia, colitis, acute hepatitis, sepsis or HIV), pregnant women, under treatment of corticosteroid, or recently recovered from surgery, trauma and myocardial infarction were excluded from the study. Among 106 patients, 59 patients had cirrhosis with HCC and the other 47 patients had cirrhosis without HCC as negative control to HCC group. All patients gave informed consent to participate in the study and the protocol was approved by ethical committee of Faculty of Medicine, University of Indonesia, Jakarta.

Test Methods

A 10 mL blood sample were collected in serum separator tube and stored at $\leq -20^{\circ}\text{C}$ until AFP and AAG measurement.

Alpha Fetoprotein Measurement

Alpha fetoprotein measurement remain the reference serum biomarker test in diagnosing HCC. The quantitative measurement of plasma AFP was performed using ADVIA Centaur AFP assay, a two-site sandwich immunoassay using

direct chemiluminometric technology, which uses constant amounts of two antibodies. The first antibody is an affinity purified polyclonal rabbit anti-AFP antibody labeled with acridinium ester. The second antibody, in the solid phase, is a monoclonal mouse anti-AFP antibody covalently coupled to paramagnetic particles. After blood sample collection, blood samples were allowed to clot adequately before centrifugation, while keeping the keep tubes stoppered and upright all times. For condition when assay was not completed within 48 hours, the specimen was refrigerated at or below -20°C . When samples were ready for the assay, 10 μL of sample was dispensed into a cuvette. As much as 50 μL of lite reagent and 250 μL of solid phase were dispensed and incubated for 7.5 minutes at 37°C . The cuvettes were then separated, aspirated, and washed using reagent water. Not less than 300 μL each of acid reagent and base reagent were dispensed to initiate the chemiluminescent reaction. A direct relationship exists between concentration of AFP present in patient sample and the amount of relative light units (RLUs) detected by the system. The results were reported in ng/mL with cutoff value of ≤ 15 ng/mL.

Alpha-1-acid Glycoprotein Measurements

Quantitative measurement of plasma AAG concentration was performed using immunoturbidimetric assay by Roche/Hitachi Cobas C system. After collection, blood samples were allowed to clot adequately for 30 minutes before centrifugation. Samples were centrifuged at 1500 g for 10 minutes before performing the assay. The serum supernatant was extracted and dispensed into sample cup. Anti- $\alpha 1$ -acid glycoprotein antibodies react with antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically using cut-off value of 3.25 g/L.

Statistical Analysis

Statistical analysis were done using SPSS 13.0. Descriptive measures were determined for each variable in every group, presented in mean \pm SD. Data comparisons between group were done using Mann-Whitney test. Spearman correlation coefficient (r) was applied to our result. A p-value < 0.05 was considered statistically significant.

For choosing the best cut off value, receiver operator characteristic (ROC) curve was generated and the Youden's index was calculated. The best cut off values had the highest Youden indices. Diagnostic performance for each marker alone (diagnostic specificity, sensitivity, positive and negative predictive values) was compared to the surrogate use of both markers (combined parallel approach) in HCC cases.

RESULTS

Baseline data of the patients can be seen in **Table 1**. There was not significant difference in the mean age between HCC and cirrhosis.

AFP serum in patients with HCC and cirrhosis (mean: 34923.96 ng/ml, SD: 80301.31 ng/ml) was significantly higher than patients with cirrhosis (mean: 31.53 ng/ml, SD: 169.98 ng/ml,

$p = 0.0005$, Mann Whitney test). As well as AFP, AAG serum in HCC patients (mean: 117.59 mg/dL, SD: 60.95 mg/dL) was significantly higher than in cirrhosis patients (mean: 66.78 mg/dL, SD: 44.84 mg/dL, $p = 0.0005$, Mann Whitney test). There was no correlation between AFP and AAG in all patients ($r = 0.172$; $p = 0.079$, correlation test) and between HCC and cirrhosis patients only ($r = -0.038$; $p = 0.773$; spearman correlation test, **Figure 1**).

To evaluate the diagnostic value of serum AFP and AAG, AUC value from ROC curve were performed (**Figure 2**). AUC value in AFP was 86.2% while AUC in AAG was 76.5%. Combination AFP and AAG produce the highest AUC value (88.1%) compared to AFP only or AAG only. At >80% specificity, the AFP sensitivity was 73% in cut off 20.45 ng/ml and

Table 1. Clinical characteristics of patients

| Clinical features | HCC + Cirrhosis (n=59) | Cirrhosis (n=47) |
|------------------------|------------------------|------------------|
| Age-year (mean±SD) | 55.53±12.01 | 55.60±11.59 |
| Gender (% male/female) | 84.7/15.3 | 55.3/44.7 |
| Hemoglobin (mean±SD) | 11.70±2.22 | 11.33±1.69 |
| Leucocyte (mean±SD) | 9538.97±6612.86 | 6625.53±2600.99 |
| Thrombocyte (mean±SD) | 232503.1±139378.0 | 123723.4±64576.5 |
| AST (mean±SD) | 171.93±216.30 | 63.74±46.46 |
| ALT (mean±SD) | 86.31±117.92 | 49.68±31.19 |
| Hepatitis B (% Y/N) | 33.9/66.1 | 32/68 |
| Hepatitis C (% Y/N) | 66.1/33.9 | 59.6/40.4 |

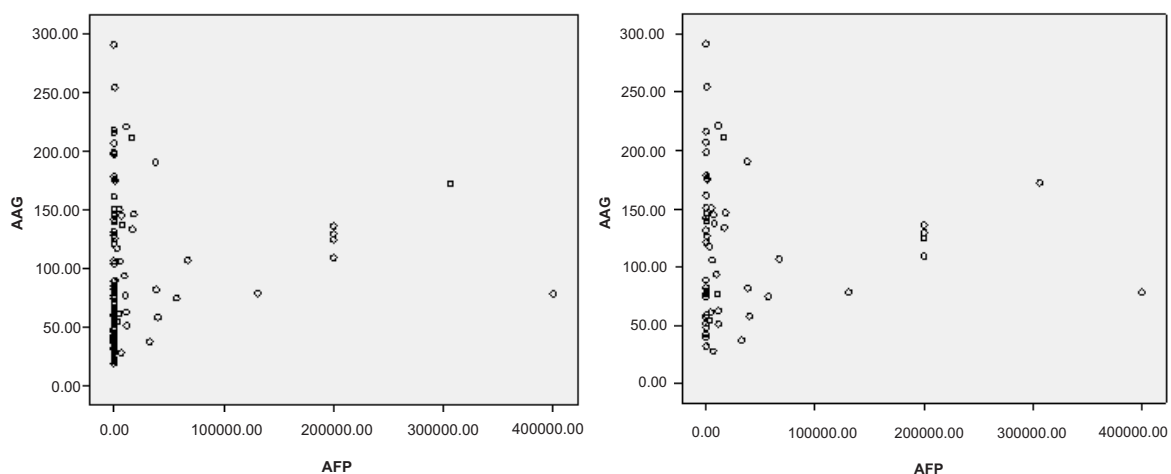


Figure 1. Scatter plot of AFP and AAG (no correlation) in all patients (left). Scatter plot of AFP and AAG (no correlation) within HCC and cirrhosis patients (right).

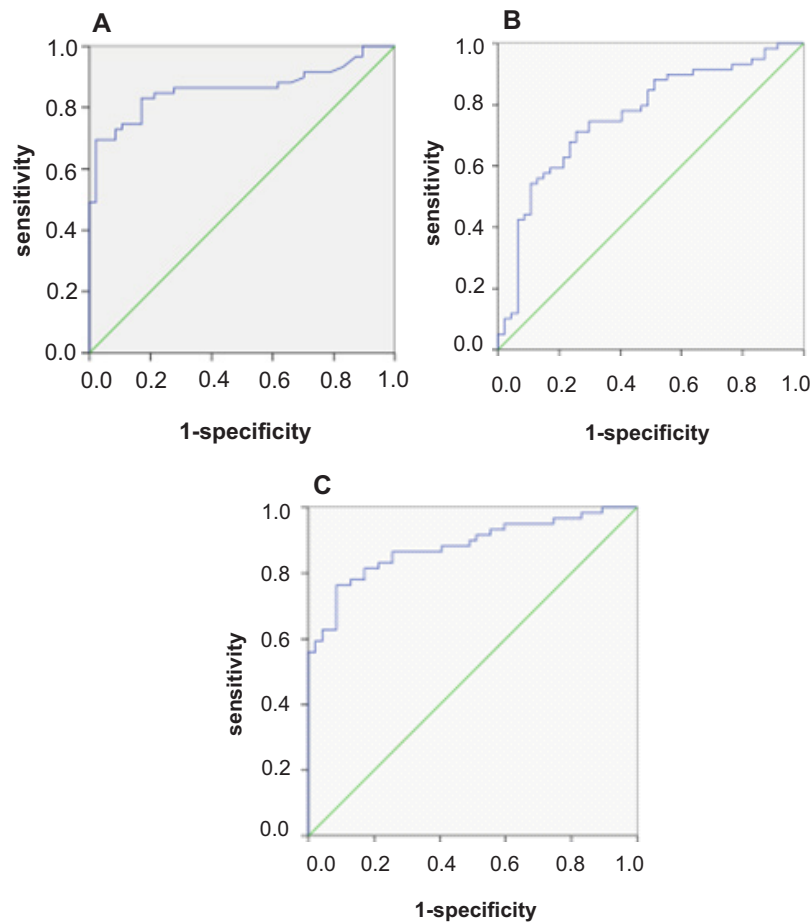


Figure 2. (a) AFP ROC curve, (b) AAG ROC curve, (c) Combination AFP and AAG ROC curve.

AAG sensitivity was 44% in cut off 129 mg/dL. Combination AFP and AAG have the highest sensitivity (85%) compared to the other (**Table 2**).

DISCUSSION

The prognosis and survival of patients with HCC is highly depend on the stage of disease at the time of diagnosis. Hence, the role of screening assay would be beneficial in detecting early stage of HCC. A review of studies showed that AFP, DCP, AFP-L3 and GPC3 can be used as serum marker for HCC.³ AFP is not a sufficient reliable marker to identify HCC patients due to its poor sensitivity.

Wang CS et. al¹⁰ found that DCP has a better diagnostic value than AFP in differentiating HCC from nonmalignant chronic liver disease, with AUC value of 85% and 73% respectively (among patients with non-cirrhotic chronic hepatitis, compensated cirrhosis, and HCC).¹⁰ On the other

hand, Nakamura S (2006) found that the AUC value of DCP was significantly smaller than AFP in tumor less than 3 cm in diameter ($p < 0.0001$) and was significantly larger than AFP in tumor greater than 5 cm in diameter ($p < 0.0001$), with chronic hepatitis or cirrhosis as control.¹¹

AFP-L3 is a fucosylated variant of AFP that reacts with lens culinaris agglutinin A and can differentiate an increase in AFP due to HCC from that in patients with benign liver disease.³ The incidence of HCC was significantly higher in patients with elevated AFP-L3% compared to those with elevated AFP.¹² Subwongcharoen S et al. found that the AUC value for diagnosis of HCC with AFP (71%) is higher than AFP-L3 (67%). In addition, the serum level of AFP was significantly different between the small mass (occupying lesser than 50% of liver volume) and large mass (occupying more than 50% of liver volume) of HCC ($p = 0.040$).¹³

Table 2. Cut-off and ROC curve analysis of AFP, AAG, and combination of AFP and AAG

| Test | Cut-off | AUC (95% CI) | P | Se | Sp | NPV | PPV |
|-----------|---------------------------------|--------------|--------|-----|-----|-----|-----|
| AFP | 20.45 ng/ml | 86.2% | 0.0005 | 73% | 92% | 73% | 92% |
| AAG | 129 mg/dL | 76.5% | 0.0005 | 44% | 92% | 57% | 87% |
| AFP + AAG | 20.45 ng/dl and/or 129 mg/dL | 88.1% | 0.0005 | 85% | 83% | 81% | 86% |

A meta-analysis of serum markers for diagnosis of HCC from 40 studies was established. AFP was considered to be the reference biomarker. It was shown that the AUC of combination AFP+DCP (0.874), were superior to the reference AFP biomarker (**Table 3**).¹⁴ GPC3 is a heparan sulfate proteoglycan anchored to the plasma membrane. It has been reported that GPC3 messenger RNA levels were increased in HCC.³ However, Ozkan H et al. study found no correlation between GPC3 levels and prognostic parameters. GPC3 was not a useful diagnostic and prognostic marker for HCC. The sensitivity and specificity of AFP (68.57, 94.55) were higher than GPC3 (61.33, 41.82).¹⁵

In this study, the AUC values of AFP (87.8%) was higher than AAG (81.4%). The differences of control group may interfere the value of AUC. In our study, the control group was patients with cirrhosis, in contrast with other studies using patients with cirrhosis and chronic hepatitis as the control.^{7,8} In the study by Chio LF and Oon CJ⁶, it was showed that AAG increased in 20% patients with cirrhosis and only 5.7% in patients with chronic hepatitis. Combination AFP and AAG obtain the highest AUC values (93%) compared to AFP alone and AAG alone. Compared to the other serum biomarker that has been mentioned previously, the combination of AFP and AAG has the best AUC value. The AUC value of DCP and AFP-L3% were lower than AFP. As well as GPC3 which has lower sensitivity and specificity than AFP. Combination AFP dan AAG have sensitivity of 78% with NPV and PPV were 73% and 91% respectively (at specificity 90%).

In the study of our group by Bachtiar et al. (2009), 220 patients were included, of which 124 had HCC and 61 (49%) of them were AFP-low HCC (AFP \leq 20 ng/mL). The remaining 96 patients, consisted of 49 with chronic hepatitis B or C and 47 with

Table 3. AUC for combination of AFP + DCP¹⁴

| Biomarker | Number of studies | AUC |
|-----------|-------------------|-------|
| AFP | 35 | 0.835 |
| DCP | 15 | 0.797 |
| AFP-L3 | 15 | 0.710 |
| AFP+DCP | 8 | 0.874 |
| AFP+AFPL3 | 3 | 0.748 |

cirrhosis, were included into control group. Their result showed the combination of AFP and AAG have higher sensitivity than AFP alone. Compared to previous one, our study is the first to specifically differentiate the use of AAG and AFP for biomarker screening in cirrhosis patients therefore it is more applicable in clinical settings since only proven cirrhosis patients were included.

Although the research has reached its aims, there were some limitations acknowledged. Due to the time and cost consideration, this research was conducted on small size of population using cross sectional design. The subject could be divided into groups based on severity of cirrhosis to study diagnostic value of AAG, AFP, and combination of both AFP and AAG on to different levels. Ideally, the number of participant would have been more evenly distributed across gender but the actual data of HCC prevalence was mainly occurred in men.

CONCLUSION

Our study showed comparison between AFP alone, AAG alone, and combination of AFP and AAG in diagnosing hepatocellular carcinoma in liver cirrhosis patients. Liver cirrhosis patients with AFP higher than 24.5 ng/ml and AAG higher than 130.3 mg/dl were highly associated with existence of HCC. Prospective studies on larger patients are required to confirm this study. Data

from this study may contribute to improve the prognosis of HCC patients by enabling early diagnosis and screening to provide early prompt, if possible, curative treatment.

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